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REVIEW

# Targeting cancer stem cells in squamous cell carcinoma

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## Abstract

Head and neck squamous cell carcinoma (HNSCC) is a highly aggressive tumor and the sixth most common cancer worldwide. Current treatment strategies for HNSCC are surgery, radiotherapy, chemotherapy, immunotherapy or combinatorial therapies. However, the overall 5-year survival rate of HNSCC patients remains at about 50%. Cancer stem cells (CSCs), a small population among tumor cells, are able to self-renew and differentiate into different tumor cell types in a hierarchical manner, similar to normal tissue. In HNSCC, CSCs are proposed to be responsible for tumor initiation, progression, metastasis, drug resistance, and recurrence. In this review, we discuss the molecular and cellular characteristics of CSCs in HNSCC. We summarize current approaches used in the literature for identification of HNSCC CSCs, and mechanisms required for CSC regulation. We also highlight the role of CSCs in treatment failure and therapeutic targeting options for eliminating CSCs in HNSCC.

**Key words:** cancer stem cells; head and neck squamous cell carcinoma; lymph node metastasis; resistance

## Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of cancer worldwide, with about 200 000 newly diagnosed cases and approximately 128 000 deaths per year.[1,2] Arising from the epithelium lining, the oral cavity, tongue, pharynx, larynx, and sinonasal tract, HNSCC is more likely to metastasize than other cancers, with around 50% lymph node metastasis

at diagnosis.[3] Currently, the standard treatments for HNSCC include surgery with adjuvant radiotherapy and chemotherapy. However, the 5-year survival rates of HNSCC patients remain below 50% and these have only marginally improved in the past few decades.

In recent years, intensive research on molecular mechanism regulating HNSCC has revolutionized the treatment for patients with this metastatic disease. Targeted therapy and immune checkpoint inhibition

have been developed and continue to be applied in HNSCC patients.[4] Despite these advancements, eradication or control of HNSCC has not been achieved, and the majority of patients will develop treatment resistance. The main root of this resistance and subsequent treatment failure is intratumoral heterogeneity, a key feature of HNSCC, comprising of a mixture of cells displaying differential degrees of sensitivity to cancer treatment. The observed heterogeneity could be the consequence of genetic alterations, epigenetic modification, environmental differences, and changes in cell properties.[5] Two models have been proposed that may explain heterogeneity, the clonal evolution model, and the cancer stem cell model. In this review, we will briefly discuss these two models of tumor heterogeneity generation and focus on characterization of HNSCC cancer stem cells (CSCs), their cellular origin, molecular regulation, and prospective therapeutic options.

## Clonal evolution versus CSCs

The heterogeneity of tumors is mainly a result of genetic or epigenetic differences between different cell types associated with tumors and tumor cells. Two models of cancer progression and metastasis progression have been proposed: the clonal evolution model and the CSC model.

### The clonal evolution model

The clonal evolution model was initially proposed by Peter Nowell in 1976.[6] Similar to Darwin's natural selection, Nowell believed that cancer is an evolutionary process driven by expansion of adapted subclones that carry selectively advantageous mutations. Accumulation of advantageous mutations in cancer cells, as a result of high genetic instability, allows them to outcompete other clones in the tissue ecosystems. Therapeutic intervention can provide potent selective pressure and allow expansion of the resistant clones. The clonal evolution model posits that all tumor cells have equal potential to form tumors, which might help to explain the progression and treatment resistance in certain cancers, such as chronic lymphocytic leukemia and acute myeloid leukemia.[7,8] And indeed, there is evidence supporting the genetic instability of solid cancer and its contribution to the genetic heterogeneity of solid tumors.[9] Using xenotransplantation, cell surface marker, and clonal cell analyses, previous studies have found that HNSCC progression follows the clonal evolution model.[10] Furthermore, another study described two different patterns of clonal dynamics in advanced head and neck cancer by assessing paired primary tumors and distant metastasis from 26 HNSCC patients and sequencing a panel of recurrently mutated genes.[11] However, more and more scientific evidence supports the hierarchical model of most solid tumors.

### The CSC model

The cancer stem cell model was proposed nearly a half century ago. According to the CSC model, CSCs are at the top of the hierarchy, symmetrically splitting to complement the CSC pool, and one-way asymmetric division produces low tumorigenic daughter cells. CSCs share similar features to normal tissue stem cells, including the ability to self-renew, proliferate, and differentiate. The main difference between the CSC model and the clonal evolution model is that it proposes a hierarchical organization of tumors. In 1994, Dick and colleagues first isolated acute myeloid leukemia stem cells based on the expression cell surface markers.[12] They found that CD34$^+$CD38$^-$ cells can give rise to a large number of colony-forming progenitor cells in transplanted SCID mice, whereas CD34$^+$CD38$^+$ and CD34$^-$ cells do not have these characteristics.[12] After identification of leukemia stem cells, the presence of CSCs was also identified in various solid tumors,[13–15] further confirming the CSC model.

Note that the clonal evolution and CSC models are not mutually exclusive in cancers. Given the randomness of obtaining additional genetic mutations, the clonal evolution model predicts that each cell can acquire the characteristics of CSCs, whereas CSCs would be expected to evolve by clonal evolution.

## Identification of HNSCC CSCs

Currently, the gold standard to define a CSC population is whether they demonstrate long-term clonal growth capabilities in functional repopulation assays, including serial transplantation into recipients or in vivo lineage tracing. Traditionally, putative CSCs in HNSCC are isolated from primary patient samples or cell lines by their unique marker expression pattern, followed by a functional limiting dilution transplantation assay. So far, multiple markers have been described in literature as putative CSC markers in HNSCC in vitro. Below we briefly discuss some commonly used markers. Table 1 provides a list of markers published for HNSCC CSCs isolation. Recently, *in vivo* lineage tracing assays have been used to make great contributions to identification of HNSCC CSCs, and we will summarize application of this technique in SCC CSCs.

### CSC isolation *in vitro*

There are several approaches to isolation of HNSCC CSCs *in vitro*: including fluorescence-activated cell sorting (FACS) approaches based on cell surface marker expression (Fig. 1), aldehyde dehydrogenase (ALDH) activity, or different efflux ability (side population); and sphere-forming assays using nonattached culture conditions.

*CD44.* Prince *et al.* first published a study showing that a HNSCC CD44$^+$ cell population displayed higher

**Table 1.** CSC markers for HNSCC CSCs isolation.

| Genes | Assay | Functions |
| --- | --- | --- |
| CD24 | Flow cytometry | CD24^{+} cells promote tumor initiation, growth and angiogenesis in HNSCC[43,44] |
| CD29 | Flow cytometry | CD29^{+} cells are related to tumor invasion ability, migration, and lymph node metastasis of HNSCC[34,173–175] |
| CD44 | Flow cytometry | CD44^{+} cells display the characteristics of CSCs in HNSCC[73,74,76,176–178] |
| CD98 | Flow cytometry | CD98^{+} cells are capable of generating tumors in nude mice and express high levels of cell cycle and DNA repair genes[179] |
| CD133 | Flow cytometry | CD133^{+} cells demonstrate CSC properties in HNSCC[36–38] |
| ALDH | ALDEFLUOR assay | ALDH^{high} population display EMT characteristics, enhanced colony forming and metastasis abilities[57,59,61] |
| c-Met | Flow cytometry | c-Met^{+} cells demonstrate chemoresistance, metastasis and CSC properties in HNSCC[49,50,180] |
| Side population | DNA dye exclusion assay | SP cells exist in HNSCC cell lines show higher metastatic potential[71] |
| Sphere | Sphere-formation assay | Spherical cells are able to survive detachment from their native microenvironment and to form cellular aggregates in an anchorage-independent manner[73] |
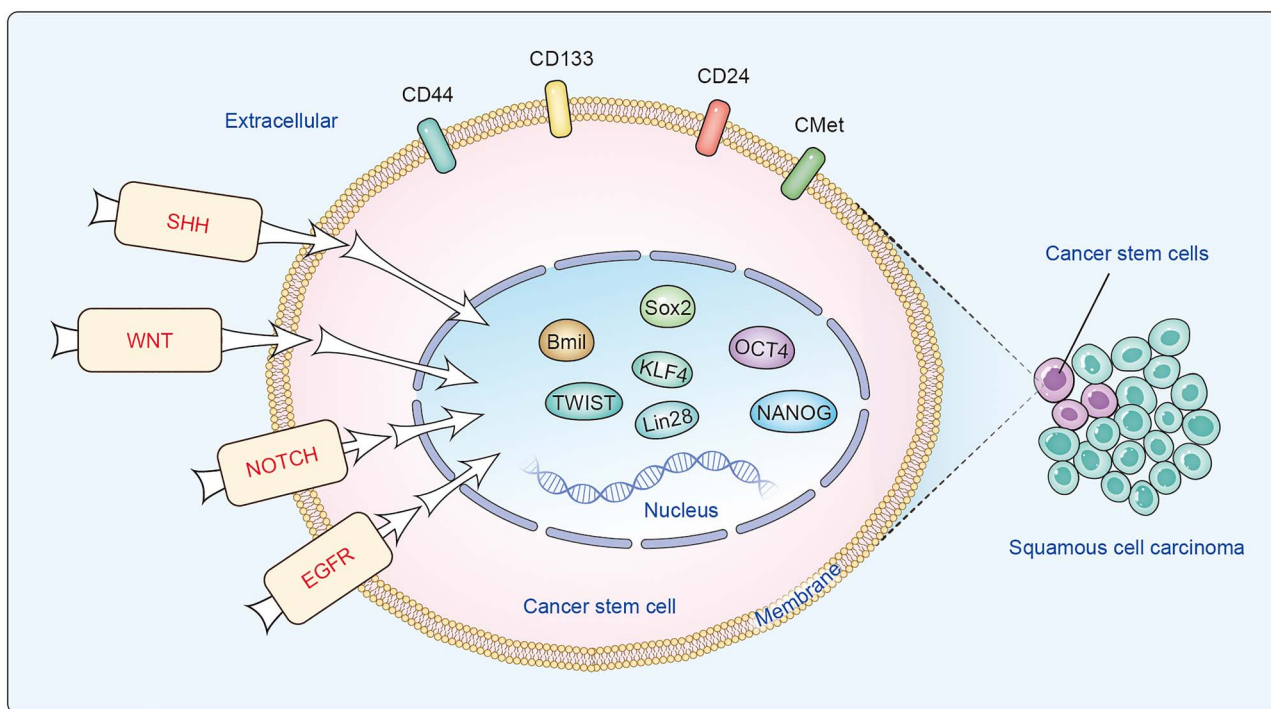


**Figure 1.** Properties of cancer stem cells (CSCs) in head and neck squamous cell carcinoma (HNSCC).

tumor-initiating ability than CD44^{−} counterparts in xenografts, proving the existence of CSCs in HNSCC.[14] Since then, CD44 has become the most well-established and commonly used CSC marker in HNSCCs.[16–19] CD44 is a type I transmembrane glycoprotein involved in intercellular interactions, cell adhesion, and cell migration.[20] There are multiple isoforms of CD44 produced by alternative RNA splicing, including the standard CD44 isoform with no variable exons (CD44s), and isoform variants with different exons of CD44 (CD44v).[21] The extracellular domain of CD44 can bind to various ligands, including hyaluronan, growth factors, cytokines, and matrix metalloproteinases.[22] CD44 is involved in activation of a variety of receptor tyrosine kinases-induced cascades, including HGF/c-Met, Src/FAK, and PI3K/AKT, which are responsible for increased proliferation and survival of cells.[23–25] Its expression is associated with locoregional recurrence, histopathological grade of malignancy, lymph node metastasis, resistance to therapy, and clinical outcome of HNSCC patients.[26] However, recent studies have also raised concern in using CD44 as a CSC marker in HNSCC. For example, systemic examination of CD44s and CD44v6 demonstrates that there is a comparable level of CD44s and CD44v6 expression between normal and benign or malignant epithelia of the head and neck.[27,28] In addition, CD44 displays constitutive expression patterns in all HNSCC cell lines, thereby reinforcing it as a reliable marker for

CSCs in HNSCC cell lines.[29,30] Therefore, the value of CD44 as a marker for the HNSCC CSCs may need to be further re-evaluated.

*CD133.* CD133, a transmembrane glycoprotein, is a well-known cell surface marker for isolation of a panel of human normal and malignant tissue stem cells.[31,32] Although CD133 is often used to isolate HNSCC CSCs, the reproducibility of using it as a marker for HNSCC CSCs is still under debate. Some studies detected no CD133 expression in freshly prepared HNSCC patient samples,[20,33,34] whereas other studies showed that cells sorted for high expression of CD133 have similar patterns of clonogenicity compared to CD133$^-$ cells.[35] In contrast, investigators reported high expression of CD133 is a CD44$^+$ cell population.[36] In addition, CD133$^+$ cells were found to have increased clonality, migratory ability, stemness, and drug resistance when compared with CD133$^-$ cells in some HNSCC cell lines.[37–40] The expression of CD133 in HNSCC prognosis also remains controversial.[41,42]

*CD24.* Another commonly used marker CD24, a cell surface glycoprotein involved in cell adhesion and metastasis, is often expressed in tumorigenic CSCs in HNSCC.[43–45] CD24 expression level is linked to cisplatin sensitivity and affects expression of critical apoptotic, stem, and drug resistance genes in HNSCC.[46] A CD24$^+$ cell population demonstrated a greater ability to self-renew and a greater resistance to chemotherapy in HNSCC.[46] Furthermore, CD24$^+$ cells can promote angiogenesis of HNSCC using a mouse model.[44] However, CD44$^{high}$/CD24$^{low}$ or CD44v3$^+$/CD24$^-$ cells show higher tumor-initiating ability, clonogenic capacity, and higher drug resistance, suggesting a distinct role of CD24 in different CSC populations in HNSCC.[47,48]

*c-Met.* c-Met, the tyrosine kinase receptor for hepatocyte growth factor (HGF), also serves as a cell surface marker for CSCs in HNSCC.[49,50] Expression of c-Met is associated with progression, invasion, angiogenesis, and metastasis of HNSCC.[51–53] The c-Met pathway also participates in cross-talk of other signaling pathways, including cellular Src kinase (c-Src), phosphotidylinsitol-3-OH kinase (PI3K), α serine/threonine-protein kinase (Akt), and mitogen-activated protein kinase (MAPK).[50,54] Sun *et al.* showed that c-Met can be used as a single marker for HNSCC CSCs and a c-Met$^+$ cell population was responsible for cisplatin-resistance and metastasis.[49] However, in retrospective studies, no consensus has been reached regarding whether expression of c-Met has an impact on overall survival or progression-free survival in HNSCC patients or not.[55,56]

*ALDH activity.* HNSCC CSCs have demonstrated elevated ALDH activity, which can allow for detoxification of aldehydes and oxidization of retinoic acid.[57–59] Thanks to the emergence of ALDEFLUOR flow cytometry assays, researchers have been able to sort live cells with high

ALDH activity (ALDH$^{high}$) and characterize the function of ALDH$^{high}$ cells in HNSCC progression.[60] ALDH$^{high}$ subpopulations in HNSCC display a more tumorigenic phenotype and resistance to radiotherapy and chemotherapy.[57,59,61] Interestingly, studies have shown that ALDH$^{high}$ HNSCC cells can sensitize autologous lymphocytes, whereas the ALDH$^{low}$ counterparts have limited ability to activate lymphocytes, suggesting the existence of unique CSC antigens in ALDH$^{high}$ CSCs.[62] To date, 19 ALDH genes have been identified within the human genome. In HNSCC, ALDH1 expression is often increased in primary isolated tumors or cell lines.[63,64] However, inconsistent results fosters uncertainty on whether ALDH1 can serve as a predictor of HNSCC prognosis.[45,65]

*Side population.* CSCs can also be obtained by isolating the side population (SP) cells based on the ability to efflux Hoechst 33342 dye. SP cells have been successfully used to identify CSC populations in a variety of solid tumors, including HNSCC.[66–69] The ability of SP cells to expel the dye lies in expression of a group of transmembrane transporters, which are involved in efflux of the chemotherapeutic drug and resistance to chemotherapy.[70] Previous reports have also shown that more SP cells exist in HNSCC cell lines with high metastatic potential than those with low metastatic potential, indicating that SP cells might be responsible for metastatic spreading of HNSCC.[71]

*Sphere-forming ability.* Sphere-forming assays have been widely used to assess the self-renewal and differentiation capability of CSCs *in vitro*. The concept of using spherical cultures to isolate CSC is based on the capacity of CSCs to survive detachment from their native microenvironment and to form cellular aggregates in an anchorage-independent manner. In HNSCC, the expression of known CSCs markers, including CD44, CD133, and ABCG2, can be dramatically enriched under serum-free medium culture conditions.[36,72] However, the ability to form spheres varies between different HNSCC samples.[73] In comparison with isolation of CSCs using cellular markers, sphere-forming assays are less specific and are often used as a validation assay for isolated CSCs.

It has become clear that a single marker is not sufficient to isolate a pure CSC subpopulation from a given tumor. Therefore, combination of multiple markers is needed for identification and characterization of CSCs. For example, ALDH$^{high}$CD44$^{high}$ has become popular as a marker for HNSCC CSCs.[59,74,75] In addition, CD44$^+$SSEA4$^+$ CSCs show greater tumorigenic capacity compared with the CD44$^+$ subpopulation, SSEA4$^+$ subpopulation, and parental cells.[76]

Because tumor initiation is one of the central characteristics of CSCs, xenografts of putative CSC populations in immunodeficient mice remain the gold standard for verification of CSC properties. In fact, application of this approach has successfully guided researchers to identify CSCs in HNSCC.[14,72,75] However, there are several drawbacks in using this xenograft model. First, the

current isolation approach inevitably causes damage of surface markers during the enzyme digestion process. Second, even orthotopic transplantation may not truly reflect the tumor microenvironment, such as stromal component and cellular architecture, of that patient's original tumor. Third, the immunodeficient mice used in the xenograft assay lack natural immunosurveillance and cell cytokines, which could impart selective forces on tumor cells and inhibit the growth of the real CSCs within a patient's tumor.

### *In vivo* identification of CSCs

Determining the cancer cells that are critical for tumor development in their native niche is important for understanding their regulation. In recent years, genetic lineage tools have been deployed in study of CSCs *in vivo*. The most widely used lineage tracing approach is the Cre-lox system, in which expression of Cre recombinase is driven by a cell-specific promoter.[77] With this approach, Cre activity can be transiently induced by administration of small molecules, allowing expression of a reporter gene. Lineage tracing studies are extremely useful to study the cellular hierarchies in cancer homeostasis.

Using genetic lineage tracing, Chen *et al*. clonally traced tumor cells *in vivo* in an unperturbed HNSCC induced by carcinogen. They found that Bmi1+ CSCs were responsible for initiation, development, and metastasis of HNSCC.[75] Interestingly, cisplatin could effectively kill proliferating cells, but it could not kill Bmi1+ CSCs, which may be the cause of HNSCC recurrence.[75] Moreover, Bmi1CreER;Rosa26DTA mice carrying HNSCC had a significantly reduced number of SCC relapses after Bmi1+ cell ablation.[75] Furthermore, in a murine cutaneous SCC model, Oshimori *et al*. showed that in the genetic lineage tracing of TGF-β the expressing cells multiplied faster and were responsible for acceleration of SCC tumor growth.[78] Boumahdi showed that Sox2+ cells and their progeny cells are the driving force of skin squamous cell carcinoma induced by DMBA/TPA.[79] Interestingly, results from *in vivo* microscopy of mouse breast tumors show undisputedly the existence of CSCs in unperturbed mammary tumors and demonstrate CSC plasticity.[80]

Although these murine models help to characterize the CSC component of SCCs *in vivo*, we must bear in mind that differences exist between mice and humans. In addition, some of the cytokines in mouse and human are not functionally equivalent, indicating that regulation of CSCs might follow different regimens in these two species.

## Regulation of HNSCC CSCs
### Stem cell factors and HNSCC CSCs

Comparison of molecular signatures between CSCs and normal stem cells uncovered a great deal of overlap between these two different kinds of cell populations.[81] Interestingly, genes associated with self-renewal, angiogenesis, migration, and anti-apoptosis were largely shared between these two stem cell populations. In particular, factors that are highly enriched in embryonic stem cells (ESCs), such as OCT4 (Octamer-binding transcription factor 4), SOX2 (Sex determining region Y-box 2), NANOG (Nanog Homeobox), KLF4 (Krüppel-like factor4), and Lin28/Let-7, are often linked to the stem-cell like feature of HNSCC CSCs (Fig. 1).

*OCT4*. OCT4 is highly expressed in ESCs and has an essential role in self-renewal and differentiation by regulating the pluripotent potential of these cells.[82,83] It has been shown that high expression of OCT4 is associated with poor survival and strongly independent prognostic effects on HNSCC progression.[72,84,85] In HNSCC, OCT4 and its target gene CIP2A were co-expressed in a CD24 positive side-population and were responsible for increased aggressiveness and radioresistance.[86] Inhibition of OCT4 expression results in reduced tumorigenic ability of HNSCC CSCs, whereas enforced expression of OCT4 in HNSCC leads to increased tumorigenicity and epithelial-mesenchymal transition (EMT) transformation of HNSCC CSCs.[87]

*SOX2*. SOX2 has emerged as a factor in maintaining self-renewal of ESC and is often enriched in HNSCC through amplification on chromosome 3q26.[82,88,89] Several studies have shown that SOX2 nuclear expression is closely associated with tumor recurrence and poor prognosis in patients with HNSCC.[90–94] The SOX2-mediated pathway is critical in HNSCC initiation and progression by regulating acquisition of CSC-like and radiochemoresistant properties in HNSCC.[89,94] Overexpression of SOX2 promotes cell proliferation via cyclin B1 expression and CSC features, including self-renewal and chemoresistance.[92] Moreover, silence of SOX2 in HNSCC CSCs substantially inhibits their self-renewal capacity, chemoresistance, invasion capacity, and in vivo tumorigenicity.[92]

*NANOG*. Similar to OCT4, a high level of NANOG is linked to poor survival and independent prognostic effects on HNSCC progression.[72,84] Expression of NANOG is elevated in both the side population and in the tumorsphere of HNSCC cell lines, implying that NANOG plays a role in regulation of HNSCC CSCs.[72,84] Indeed, knockdown of NANOG can effectively block CSC-like properties and increase drug sensitivity and apoptosis of HNSCC CSCs.[95] Interestingly, NANOG protein stability can be regulated by human protein kinase C$\varepsilon$ via phosphorylation at T200 and T280 residues.[96] Inhibition of T200A or T280A phosphorylation in NANOG can lead to decreased cell proliferation, colony formation, invasion, migration of the CSC population in HNSCC cells.[96]

*KLF4*. KLF4 is a relatively large family of zinc finger transcription factors belonging to sp1-like transcription factors. The role of KLF4 remains controversial in HNSCC. It has been shown that expression of KLF4 displays an inconsistent pattern in HNSCC.[97,98] Although KLF4 protein expression is decreased in the majority of HNSCC

patient samples, there is still persistent KLF4 expression detected in some HNSCC.[97] HNSCC with high KLF4 expression is often associated with a lower disease-specific survival, whereas ectopic KLF4 expression promotes HNSCC progression.[88] However, using a genetic mouse model, a study has demonstrated that conditional knockout of Klf4 expression in the oral epithelium promotes development of malignant oral SCC lesions, suggesting a potential tumor suppressor role of Klf4 in HNSCC.[99]

*LIN28/Let7.* As a well-known RNA-binding protein, LIN28 plays a critical role in regulating the balance between stemness and differentiation in ESCs via regulation of the microRNA Let-7.[100] Lin28 is highly expressed during embryogenesis and is critical for the determination of stemness state in multiple tissue lineages.[101] In contrast, Let-7 often serves as a tumor suppressor in a variety of cancers, most likely through targeting oncogenes, such as RAS or HMGA2.[102,103] In HNSCC, dysregulation of Let-7 is associated with patient clinical outcomes.[104] Furthermore, Let-7 expression is dramatically decreased in ALDH^high putative CSCs compared to ALDH^low population.[105] Functional assays further confirm that Lin28B-Let7 is required for Oct4 and Sox2 expression, and for the self-renewal properties of HNSCC CSCs.[103] Specifically, targets of Let7, including ARID3B and HMGA2, can directly regulate Oct4 and Sox2 expression via binding to their promoter.[103]

*BMI1.* BMI1 (B lymphoma Mo-MLV insertion region 1 homolog) is a polycomb group (PcG) protein that plays an important role in the self-renewal and epigenetic regulation of normal and cancer stem cells. BMI1 serves as a key component of the polycomb repressive complex 1 (PRC1), which represses gene expression through monoubiquitination of histone H2A.[106,107] High BMI1 often correlates with advanced stages, aggressive clinicopathological behaviors, stem cell-like properties, drug resistance, and poor prognosis in HNSCC.[14,59,75,108] Down-regulation of BMI1 can reduce the cell sphere and colony formation.[109] Pharmacological targeting of BMI1, using a small molecule inhibitor, dramatically impaired tumorigenesis in HNSCC.[75,110,111]

## Signaling pathway and HNSCC CSCs

Signaling pathways, including Sonic Hedgehog (SHH), Wnt, epidermal growth factor receptor (EGFR), and Notch, that control normal stem cell self-renewal and differentiation are often aberrantly activated in HNSCC CSCs (Fig. 1). Therefore, identification of the crucial pathways necessary for CSC maintenance may provide important therapeutic targets for HNSCC.

*SHH pathway.* The SHH signaling pathway is an important mechanism for embryonic development and homeostasis of mature tissues.[112] The pathway is activated on binding of SHH to the PTCH receptor, which in turn derepresses the Smoothened (SMO)

transmembrane receptor.[113] Activation of SMO then triggers the SHH signaling cascade via recruitment and activation of GLI family transcription factors, including GLI1, GLI2, and GLI3.[113] Previous studies have shown that SHH signaling is upregulated in various CSCs, including breast cancer, liver cancer, brain tumors, and gastric cancer.[114–117] In HNSCC, elevated levels of GLI, PTCH1, SMO, and SHH can be detected in tumor compared with normal oral mucosa.[118] In addition, expression of GLI1 is associated with lymph node metastasis, recurrence, clinical stages, and poor prognosis. Consistently, blockage of the SHH signaling pathway leads to inhibition of tumor growth and angiogenesis in HNSCC.[119] Moreover, inhibition of GLI3 in HNSCC cell lines leads to a decrease of CD44^high CSCs and sphere-forming ability by inhibition of OCT4 and BMI1 expression.[17]

*WNT pathway.* The WNT signaling pathway is an evolutionarily conserved pathway critical for stem cell self-renewal and fate determination.[120] Based on the dependency of β-catenin, the WNT signaling pathway can be divided into two, namely canonical and non-canonical pathways. Activation of the canonical WNT/β-catenin signaling pathway requires binding of WNT ligands to Frizzled and its co-receptor, lipoprotein receptor-related protein 5 or 6 (LRP 5/6), which then recruits the β-catenin degradation complex and allows accumulation of β-catenin in the cytoplasm.[121] Consequently, β-catenin will be translocated into the cell nucleus to promote transcription of downstream targets via binding to the N-terminal of T-cell factor/lymphoid enhancing factor (TCF/LEF) transcription factors.[121] Previous studies have demonstrated an important role of the WNT/β-catenin signaling pathway in maintenance of HNSCC CSCs. For example, treatment of WNT activator or WNT inhibitor can alter the CSC proliferation rate in HNSCC, while this treatment shows minimal effect on the parental HNSCC cells, suggesting a role for the WNT/β-catenin signaling pathway in HNSCC CSCs.[122–124] In addition, a recent study showed that Wnt activation in HNSCC increased the properties of CSCs, such as sphere formation and invasiveness.[125]

*EGFR pathway.* The EGFR is a transmembrane protein that is activated via binding of its specific ligands, including epidermal growth factor (EGF) and transforming growth factor α (TGFα), which triggers activation of an intracellular signaling cascade to control cell growth, differentiation, and survival.[126] Elevated levels of EGFR are often observed in HNSCC, and increased activity of the EGF pathway has been associated with resistance to treatment and poor clinical outcome.[127] Results have shown that high expression of EGFR promotes a higher functional proportion of ALDH^high CSCs in a high aggressive human papillomavirus-16 (HPV-16) + cell line UM-SCC-104.[128] In addition, the CSC cell surface marker CD44 can interact with EGFR to enhance progression of HNSCC.[129]

*NOTCH pathway*. Notch signaling plays an essential role in a variety of stem cell processes, such as cell proliferation, differentiation, and self-renewal.[130] Four Notch receptors (Notch 1–4) and five Notch ligands (Jagged-1, Jagged-2, Delta-1, Delta-3, and Delta-4) have been reported in mammals.[131] When the Notch receptor is activated, the Notch intracellular domain (NICD) will be released from the plasma membrane and translocates into the nucleus.[131] Together with the CSL transcription factors, NICD will then induce the expression of its target genes, such as Hes-1 and Hey-1.[131] Inhibition of NOTCH1 by antibody or inhibitor is able to repress tumor growth and CSC function in multiple tumors.[132,133] In addition, NOTCH1 inhibition can impair the tumorigenesis and CSC self-renewal of HNSCC using a xenograft model.[134] However, studies have also reported that Notch1 might serve as a tumor-suppressor gene in HNSCC.[135,136]

## EMT and HNSCC CSCs

EMT is a process in which epithelial cells change their morphology, lose their polarity and acquire the migratory properties of mesenchymal cells. During EMT, epithelial cells gradually lose expression of intercellular adhesion molecules, such as E-cadherin and keratin, and gain expression of vimentin, N-cadherin, and fibronectin.[137] EMT is mainly mediated by a core set of transcription factors, including SNAIL, SLUG, TWIST1, ZEB1, and ZEB2.[138] Aberrant regulation of these EMT transcription factors can induce cancer cell plasticity and promote tumor initiation and metastatic spread. In addition, EMT transcription factors are able to induce many other traits, such as cell survival and stem cell-like features.[139] Mani *et al.* first provided experimental evidence that induction of EMT leads to upregulation of stem cell markers in breast cancer cells.[140] Since then, accumulating evidence in various solid tumors supports that EMT induction not only promotes tumor cell invasion and metastasis, but also enhances drug resistance and enriches CSCs.[141–143]

In HNSCC cell lines, studies have shown that ALDH$^{high}$ putative CSCs display EMT characteristics and enhanced colony forming abilities.[144] In addition, two different types of CSCs displaying distinct phenotypes exist in HNSCC, including CD44$^+$ ESA$^{low}$ EMT CSCs expressing EMT markers and CD44$^+$ ESA$^{high}$ non-EMT CSCs displaying epithelial characteristics.[145] However, only the ALDH1$^+$ EMT CSCs in HNSCC are able to metastasize and form new tumors.[145] Hence, some researchers suggested that high expression of EMT transcription factors, stemness-related factors, and ALDH1$^+$ cells should be used as conditions for identifying CSC of HNSCC.[144,146] Studies have found simultaneous high expression of EMT-related genes (SNAIL, SLUG, TWIST, Vimentin, and Fibronectin) and stemness-related genes (OCT4, NANOG and BMI1) in metastatic cell lines of HNSCC.[144,147] BMI1 can be directly regulated by TWIST, and synergistically promotes EMT and the tumor-initiating capability of HNSCC with TWIST.[147] In addition, overexpression of Twist results in upregulation of HNSCC CSC markers, such as ALDH and CD133 in HNSCC cell lines.[111]

Recently, studies indicated that EMT is not a two-level process from epithelial to complete mesenchymal state, but gradual events passing through different intermediate hybrid states. Different stages of EMT show different cellular plasticity, invasiveness, and metastasis potential,[148] which further supports the heterogeneous nature of EMT. Furthermore, different sources of cells may affect whether or not cells undergo EMT. For example, in murine cutaneous SCC, hair follicle-derived cancer cells are more prone to undergo EMT than interfollicular epidermis-derived cancer cells. And genes of EMT in hair follicle lineages are regulated by transcription factors related to hair follicle stemness and differentiation.[149] These findings may provide an explanation why some types of cancer cells retain an epithelial organization rather than mesenchymal traits when metastasis occurs.[143] Metastatic cancer cells may be located in a different type of intermediate hybrid state of EMT or these cancer cells may not easily undergo the EMT process. Therefore the findings do not dispute the hypothesis that EMT is necessary to maintain the CSC phenotype.

## HNSCC CSC and drug resistance

There are several mechanisms of drug resistance in cancer, including reduced drug aggregation, changes in drug target, reduced apoptosis, and increased DNA damage repair. According to the CSC hypothesis, the stem cell-like properties of CSCs help tumors to resist chemotherapy agents. However, studies have also shown that treatment with chemotherapy drugs can increase stemness in multiple solid tumors.[150–152] To study the chemoresistance of HNSCC CSCs, researchers need to take into account the specific markers that are used for isolation of CSCs, because CSC markers may possess the molecular features to antagonize chemotherapy.

For example, CD44 can interact with hyaluronan to promote phospholipase C-mediated intracellular Ca$^{2+}$ mobilization in order to prevent apoptotic effects caused by cisplatin.[126] In addition, hyaluronan triggers an interaction between CD44v3 and OCT4-SOX2-NANOG, which in turn induces expression of miR-302 and survival proteins (cIAP-1, cIAP-2, and XIAP), thereby allowing cells to become tolerant to cisplatin.[153] Another marker, CD133, is critical in chemoresistance by positively regulating expression of CSC-related genes.[154] Chemotherapeutic drugs, such as cisplatin and erlotinib, can increase the level of reactive oxygen species (ROS) and lipid peroxidation-derived aldehydes.[155] It is reasonable to suspect that high activity of ALDHs, which catalyze oxidation of aldehydes, can help in metabolizing these reactive aldehydes and reducing oxidative stress in HNSCC CSCs. Indeed, targeting the activity of ALDH using small molecule inhibitors, greatly impairs the cisplatin resistance in HNSCC.[156] In HNSCC, c-Met-expression cells exhibit cisplatin-resistant capacity.[49] Blockage of c-Met with ARQ 197 and crizotinib, two small molecule inhibitors of c-Met, results in reduced tumorigenesis in HNSCC both *in vivo* and *in vitro*.[157] These studies indicate

that markers for isolating CSCs are also responsible for drug resistance in CSCs.

Stem cell factors also take part in chemoresistance of HNSCC CSCs. For instance, SOX2 can promote cisplatin resistance of HNSCC CSCs through ABCG2 (ATP-binding cassette super-family G member 2) expression.[92] Upregulation of OCT4 and NANOG has also been associated with chemoresistance in HNSCC.[158] Drug resistance mediated by a HNSCC CSC marker is often involved in activation of the PI-3 kinase/AKT signaling pathway, in which increased phosphorylation of AKT inhibits activation of the apoptotic effect.[63]

A recent study suggests the existence of plasticity between CSC and non-CSC populations.[23] This has led researchers to wonder whether targeting both CSC and non-CSC populations will be needed for better treatment. A study by Chen *et al.* provided experimental evidence demonstrating reduced primary tumor volume and lymph node metastasis through the means of targeting mouse HNSCC with PTC-209 (Bmi1 inhibitor) and cisplatin.[75]

## Clinical and therapeutic implications of HNSCC CSCs

Despite the advances in immune-checkpoint inhibitors in HNSCC, early studies from clinical trials show limited efficacy with monotherapy in HNSCC compared to traditional chemotherapy. Thus, more effective approaches are required for HNSCC. Targeting CSCs could improve the efficacy of cancer therapy because CSCs can resist chemotherapy and radiotherapy, leading to a population of cancer cells left behind to continue to grow and spread. Although intensive efforts have been made towards targeting CSCs in different tumor types, most of them showed limited effect, probably because of the failure to identify the *bona fide* CSCs population. Besides, researchers also need to be aware that CSCs are heterogeneous based on the genetically unstable nature of cancer cells. Another point of note is that CSC-targeted therapy is possibly toxic toward normal stem cells. Here, we discuss some recent preclinical and clinical progress in targeting HNSCC CSCs.

### Targeting cell surface markers

Cell surface markers have been explored previously as possible pharmaceutical targets of CSCs in HNSCC. Targeting CD44v6 using chimeric monoclonal antibody U36 in patient or monoclonal antibody BIWA in xenograft results showed that this approach can reduce tumor size or can be useful for fluorescence-guided surgery.[159,160] Furthermore, Yu *et al.* showed that targeting CD133 can overcome the chemoresistance in HNSCC-derived SP cells, suggesting that cell surface makers are promising targets for CSC inhibition.[39]

### Targeting tyrosine kinases

Receptor tyrosine kinases promote tumor progression in HNSCC and are an attractive therapeutic target.

Cetuximab is a US Food and Drug Administration (FDA)-approved monoclonal antibody targeting EGFR in HNSCC. Treatment with cetuximab also promotes differentiation of CSCs and impairs resistance of CSC to chemo/radiation.[161] Also, a newly discovered gamboge derivative compound 2 showed effective inhibition of HNSCC by targeting a CSC population through EGFR tyrosine phosphorylation.[162] However, another study has demonstrated that the EGFR-targeted agents, such as cetuximab and erlotinib, show only modest effects on tumor control of HNSCC.[161]

### Targeting stem cell factors

Progression of HNSCC is associated with Bmi1-positive CSCs, which are responsible for tumor invasion, drug resistance, and lymph node metastasis. Hence, Bmi1 serves as a potential CSC therapeutic target for HNSCC. Inhibition of BMI1 by the small molecule inhibitor PTC-209, enhances the therapeutic effect of cisplatin and inhibits lymph node metastasis in HNSCC.[75] In addition, a study has shown that the flavonoid derivative 2-(3-hydroxyphenyl)-5-methylnaphthyridin-4-one (CSC-3436) can effectively inhibit EMT, cancer stemness, and migration/invasion abilities via downregulation of the Twist/Bmi1-Akt/β-catenin pathway.[111] Heat shock protein 90 inhibitors, KU711 and Ku757, are able to effectively target CSCs in HNSCC and inhibit the expression of BMI1 and EMT.[163]

### Targeting immune checkpoint inhibitors

To date, various immunotherapy approaches have been developed to target HNSCC, including vaccines, T cell infusion, immune checkpoint inhibitors, and tumor-specific monoclonal antibodies.[164] In 2016, anti-PD1 antibodies (nivolumab and pembrolizumab) were approved by the FDA for treatment of HNSCC in the second-line setting. In these pilot studies, results have demonstrated that nivolumab and pembrolizumab can improve overall survival of HNSCC compared with traditional chemotherapy.[4,165,166] However, response rates to nivolumab and pembrolizumab in HNSCC remain low, ranging only from 13% to 20%, whereas survival has improved in one of 10 patients who received these therapies. The heterogeneous nature of HNSCC might be the key to the unresponsiveness and resistance to immunotherapy. A recent study using a mouse model has shown that TGF-β responsive CSCs are responsible for resistance of immunotherapy and cancer recurrence through selective expression of CD80, a well-known immune checkpoint protein,[167] supporting the notion that targeting CSCs might improve the current immunotherapy of HNSCC.

### Targeting HNSCC CSCs by natural compounds

In recent decades, natural compounds have been recognized more and more as useful drugs to target CSCs in multiple cancer types, including HNSCC.[168–172,174,175] For example, BE-43547A2, which belongs to the naturally

occurring cyclic depsipeptides, can selectively target pancreatic CSCs.[178] Researcher have also found several naturally derived compounds that can ablate CSCs in melanoma more effectively.[177] In addition, curcumin, a natural product extracted from *Curcuma longa* plants, can suppress stemness of HNSCC CSCs through inhibition of RXRα.[178]

## Concluding remarks and future perspectives

CSCs constitute a small portion of tumor cells and display the ability to renovate new tumors. Hence, targeting the CSC population has become an effective method of cancer treatment, although CSC-targeting therapeutics are still in their infancy. Although intensive studies have been performed to investigate the characteristics and regulation of CSCs in HNSCC, many of these were based on *in vitro* data and none have gone through the early clinical stages. As previously mentioned, for development of new strategies for targeting CSCs in HNSCC, we need a better understanding of CSC properties and their druggable targets. Furthermore, it is important to realize that precision medicine might provide great value for treatment of different patients who show different CSC regulation.

## Acknowledges

## Conflict of interest

None declared.

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